To study the role of Mycobacterium tuberculosis cell wall associated glycoproteins immunomodulation inside the macrophages.

Dissertation submitted in partial fulfillment for the degree of

Master of Science in Applied Microbiology

Submitted By

UTSAV MUKHERJEE

1662021

School of Biotechnology (Campus 11)

KIIT (Deemed to be University)

Bhubaneswar, Odisha, India

Under the Supervision of

Prof. (Dr) Avinash Sonawane

School of Biotechnology, KIIT (Deemed to be University)

School of Biotechnology
Kalinga Institute of Industrial Technology (KIIT)
Deemed to be University
(Established U/S 3 of UGC Act, 1956)
Bhubaneswar, Odisha, India

CERTIFICATE

This is to certify the dissertation thesis entitled as "***To study the role of Mycobacterium tuberculosis cell wall associated glycoproteins immunomodulation inside the macrophages***" Submitted by **Utsav Mukherjee** in partial fulfilment of the requirement for the degree of Master of Science in Applied Microbiology, KIIT School of Biotechnology, KIIT Deemed to be University, Bhubaneswar bearing Roll No. **1662021** & Registration No. **16647651473** is a *bona fide* research work carried out by him under my guidance and supervision from 19th December 2017 to 12th May 2018.

Supervisor name and signature

School of Biotechnology
Kalinga Institute of Industrial Technology (KIIT)
Deemed to be University
(Established U/S 3 of UGC Act, 1956)
Bhubaneswar, Odisha, India

CERTIFICATE

This is to certify that the dissertation thesis entitled as "***To study the role of Mycobacterium tuberculosis cell wall associated glycoproteins immunomodulation inside the macrophages***" submitted by **Utsav Mukherjee**, *Roll No.***1662021**, *Registration No*. **16647651473** to the School of Biotechnology, KIIT Deemed to be University, Bhubaneswar-751024, for the degree of Master of Science in Applied Microbiology is his original work. The thesis is written based on the results of the experiments and investigations carried out independently by him during the period from 19th December 2017 to 12th May 2018 of study under my guidance.

Further, it is also to certify that the above mentioned work has not been previously submitted for the award of any degree, diploma, or fellowship in any Indian or foreign University.

Date:

Place: Supervisor name and signature

DECLARATION

I hereby declare that the dissertation thesis entitled "***To study the role of Mycobacterium tuberculosis cell wall associated glycoproteins immunomodulation inside the macrophages***" submitted by me, for the degree of Master of Science to KIIT Deemed to be University is a record of bona fide work carried by me under the supervision and guidance of **Prof. (Dr) Avinash Sonawane, Dean, KIIT School Of Biotechnology, Bhubaneswar, Orissa, India.**

Date:

Place: Student Name & Signature

CHAPTER 1

ABSTRACT

Mtb glycoproteins are potent immunomodulators which modulates the host immune response in favor of its intracellular persistence. In this report we aim to characterize a functionally unknown *Mtb* gene, encoding for an acetyltransferase (ACTase). We observed that the ectopic expression of ACTase in *Msm* (the non-pathogenic strain of mycobacteria) resulted in increased intracellular persistence via inhibiting the production of oxidative radicals. The inhibition of reactive oxygen species (ROS) formation inside the host thereby inhibited the autophagy pathway (via over-expression of mTORC-1 and autophagy adaptor molecules like p62 and NBR; and inhibition of p-ULK-1, p-AMPK, ATG 5/7, Beclin-1 and LC3 expression). The transcriptional regulator like TFEB, Nrf2 and Keap-1 were also observed to be modulated in the MsmACTase infected cells. Scavenging of the ROS formation was found to be modulated by the induction of peroxisome turnover, as blocking the peroxisome function increased the ROS level in the cells and upregulated the autophagy pathway.

ACKNOWLEDGEMENTS:

Words are never enough to express heart-felt gratitude. It has been a privilege and honor to thank **School of Biotechnology, KIIT Deemed to be University** for granting me the opportunity to carry out the dissertation as a part of my partial fulfillment of my M.Sc degree. Any accomplishment requires the effort of many people and this work is no different. I take this opportunity to express my gratitude to the people who have a core participation in the successful completion of this project.

I offer my heartfelt gratitude to my supervisor and guide of the project, **Prof. (Dr) Avinash Sonawane**, **Dean, KIIT School of Biotechnology**, Bhubaneswar, Orissa for providing me an opportunity to avail facilities for the completion of my project and for his constant support and illuminating guidance through the project. Without his involvement and supervision, I would not be able to complete this project.

I would like to express my sincere thanks to **Ms. Geetanjali Ganguly**, who helped me a lot in the lab and shared her valuable experiences with me. A special mention about her continuous guidance during this period, innovative ideas, scientific discussions were the true reason behind the work being a success. I am indebted to her, as without her continuous guidance this project would not have materialized.

In, particular I would like to extend my gratitude and acknowledgement to **Mr. Avinash Padhi, Ms. Srabasti Sengupta, Mrs. Ishani Das, Mr. Kaliprasad Pattanaik, Ms. Ananyaashree Behera, Ms. Manaswini Jagadeva, Mr. Mainak Biswas, Ms. Barsa Nayak, Ms. Deepmala Modanawal, Ms. Shomika Sengupta, Mr. Sumanta Naik and all other IL members** for their guidance and immense support in timely and successful completion of the project work.

In my daily work I was blessed with a friendly and cheerful group people from other labs who willingly helped me with their abilities. Their unfailing co-operation and friendly nature would be remembered.

Sincere thanks to my friend Priya Ghosh who willingly helped me throughout the work.

I am grateful to **Dr. Mrutyunjay Suar**, Director, KIIT School of Biotechnology for allowing me to undertake this training which has been a great learning experience.

Last of all, I would like to thank all my friends, who morally supported and encouraged me a lot throughout this duration.

In the end I would like to thank almighty God and my parents, Mr. Aniruddha Mukherjee and Mrs. Shyamali Mukherjee, for giving me strength every day, for the guidance and the good health, for the graces and blessings, which help me to perform my task.

TABLE OF CONTENTS:

Page No.

1. ABSTRACT………………………………………………… 6

2. INTRODUCTION……………………………………….. 12-13

3. REVIEW OF LITERATURE…………………………… 15-24

4. AIM AND OBJECTIVES………………………………. 26

5. METHODS……………………………………………. 28-34

6. RESULTS…………………………………………… 36-45

7. DISCUSSION……………………………………….. 47-48

8. CONCLUSION…………………………………….. 50

LIST OF ABBREVIATIONS

TB: Tuberculosis

M.tb: *Mycobacterium tuberculosis*

WHO: World health organization

NMR: Nuclear magnetic resonance spectroscopy

IL: Interleukin

CFU: Colony forming unit

µg: Microgram

LIST OF THE FIGURES IN REVIEW OF LITERATURE

Figure 1: Ziehl-neelsen staining of *Mycobacterium tuberculosis*

Figure 2: Colony morphology of *M. tb* H37Rv

Figure 3: A comparative diagram of Mycobacterial cell wall and cell wall containing glycoproteins

Figure 4: Mycobacteria bacilli infecting a susceptible healthy host

Figure 5: Mycobacterial cell wall structure

Figure 6: Production of cellular ROS compounds through ETC cycle inside mitochondria

Figure 7: β-oxidation of VLCFA to short chain fatty acids

Figure 8: Formation of autolysosome through the formation of autophagosome

Figure 9: Classical autophagy pathway

CHAPTER 2

INTRODUCTION

Tuberculosis (TB) one of the most fatal pulmonary disease affecting one third of the world's population, commonly caused by *Mycobacterium tuberculosis (Mtb).* As accounted in WHO 2016 report 6.3 million instances of TB were accounted for which is comparable to 61% of the evaluated rate of 10.4 million. Most of the estimated number of incident cases occurred in the WHO south-east Asia region (45%), the WHO African region (25%) and the WHO Western Pacific Region (17%) and smaller portions of cases occurred in the WHO Eastern Mediterranean Region (7%), the WHO European Region (3%) and the WHO Region of the Americas (3%). According to the WHO 2016 report India accounted for about 27 million infected individuals out of which 4.5 lakh people died. The high number of TB incidence is due to the drug resistant property of the *M. tb*. These are differentiated into two different categories those are- MDR-TB (Multi-drug resistant Tuberculosis), which are resistant to two most popular anti-TB drugs those are isoniazid and rifampicin, and XDR-TB (Extensively-drug resistant Tuberculosis) which shows resistivity towards any of the fluoroquinolones (such as levofloxacin or moxifloxacin) and to at least one of the three injectable second-line drugs (amikacin, capreomycin or kanamycin).

Mycobacteria exist as both pathogenic and non-pathogenic species. The fundamental difference between the two species is that the pathogenic bacilli seize the maturation of the phagosomes, however the non-pathogenic strain is killed via the phagolysosome fusion. Mycobacteria are 2-4 µm in length, characterized as non-motile, acid-fast, obligate aerobe. Mycobacteria can be distinguished from the rest of the Actinomycetes on the basis of their ability to synthesize mycolic acids. The mycolic acids layer in the cell wall plays a crucial role in drug resistance.

Upon infection with mycobacteria, the bacilli are phagocytosed by the macrophages. Here the bacteria are exposed to antibacterial effecter molecules such as reactive nitrogen species (RNS) and reactive oxygen species (ROS). It has been well established that at physiological low levels- reactive oxygen species(ROS) serve as signaling messenger for various biological responses, including gene expression, cell proliferation, angiogenesis, innate immunity, programmed cell death and senescence [1]. ROS and RNS are produced by the host enzymes (such as NADPH oxidase, Superoxide dismutase, Catalase and Xanthine Oxidase) which play a critical role in controlling *Mtb* pathogenesis [2-3]. However, the intracellular bacilli have developed strategies to combat the anti-bacterial activity of ROS and RNS [4]. It has been

reported that Mel2 locus in *Mtb* genome plays a significant role in the protection against ROS, *Mtb* was susceptible to ROS [5].

It has been reported that the ROS and RNS are intracellular signal transducers for the lysosomal degradative process known as Autophagy. This plays essential functions in cellular innate immunity, particularly in the clearance of intra-cellular bacteria and also in the maintenance of cellular homeostasis [6]. Autophagy can be categorized into three major groups: 1. Chaperone mediated autophagy (CMA), 2. Microautophagy, 3. Macroautophagy [7]. Autophagy begins with the sequestration of cytoplasmic components which includes dysfunctional cell organelles or unused proteins etc, inside a double-membrane bound organelle commonly known as autophagosomes, lately these autophagosomes fuse with the lysosomes to form the autolysosomes and acquire lysosomal hydrolases which results in the degradation of its engulfed constituents [8].

Mitochondria is one of the prime site to study intracellular ROS production, however the single membrane structure 'peroxisomes' are also reported to play a crucial is redox and cellular homeostasis. These organelles comprises of different enzymes which participate in a wide range of cellular processes including the metabolism of fatty acids, lipids and generation as well as detoxification of hydrogen peroxide [9]. The principle functions of peroxisomes are to regulate many metabolic pathways that are involved in lipid metabolisms which include β-oxidation of very long chain fatty acids (VLCFA), synthesis of ether glycerolipids, formation of bile acids and cholesterols and catabolism of purines and amino acids [10]. Selective degradation of peroxisomes is termed as Pexophagy. In this process peroxisomes are selectively sequestered into the specialized autophagosome and finally degradation through the formation of phagolysosomal complex. Here in this report we aim at studying the role of an *M.tb* gene encoding for acetyltransferase (ACTase) in redox homeostasis via peroxisome formation and pexophagy.

CHAPTER 3

REVIEW OF THE LITERATURE

Mycobacterium tuberculosis the etiological agent causing TB belongs to the genus *Mycobacterium,* family of *Mycobacteriaceae* in the order Actinomycetes. *Mycobacterium tuberculosis* was first identified by Robert Koch as the organism responsible for causing TB among humans.

Some of the well known *Mycobacteria*:

Mycobacterium tuberculosis is a member of *Mycobacterium tuberculosis* complex (MTBC). Besides Mtb the other member of the complexes are *Mycobacterium bovis, Mycobacterium leprae, Mycobacterium smegmatis, Mycobacterium avium, Mycobacterium fortuitum, Mycobacterium africanum, Mycobacterium marinum, Mycobacterium microti. Mycobacterium smegmatis* (*Msm*) is the non-pathogenic strain of Mycobacteria, which have been used as a surrogate model of all the experiments regarding the mycobacterium [11], because of its rapid generation time (3-4h) in comparison to the slow generation (15-18h) of pathogenic *Mtb*. Interestingly *Msm* also shares more than 2000 homologs with *M. tuberculosis* [12].

Staining and colony features of Mycobacterium:

Mycobacteria are to some extent similar to the Gram positive bacteria, however since they lack a true outer layer and peptidoglycan so these also share some similarities with Gram negative bacteria, as a consequence *Mycobacteria* cannot retain the Gram stain. It is known to be stained by carbol fuchsin (Zeihl-Neelsen staining) since they are acid fast and retain the stain even after acid alcohol treatment.

Due to the presence of the mycolic acid in the cell wall of *Mycobacteria* it tends to grow in the form of a clump with irregular linings on a media plate.

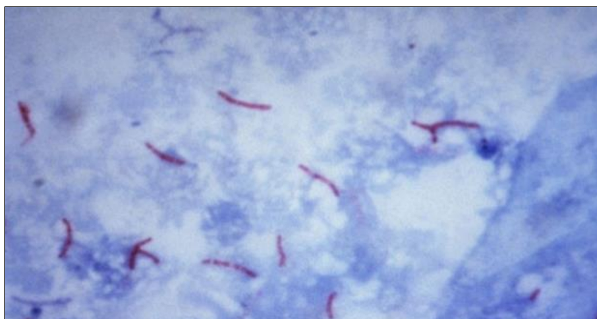


Figure 1

Figure 2

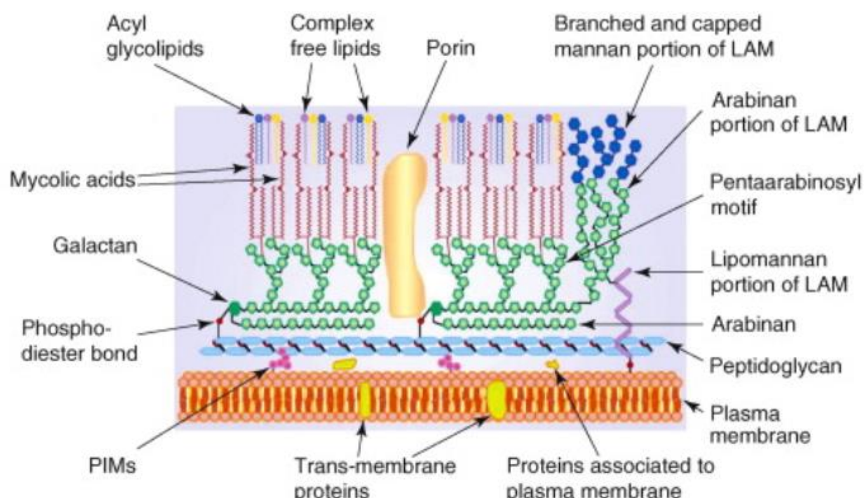


Figure 3

***Mycobacteria* pathoegenesis and its relativity with growth rate:**

Extensive studies with the help of NMR and mass spectroscopy has helped in elucidating the Mtb cell wall structure to some extent. The cell wall is made to two layers. The inner component contains peptidoglycan which are linked via linker unit (l-Rha-o-GlcNAc-P) to a linear galactofuran which is attached to branched arabinofuran and these are subsequently attached to mycolic acid [13], C60- C90 long fatty acid which is about 30% -40% of the total weight are important for pathogenecity and survival of mycobacteria, Mycolic acid also occurs as trehalose di mycolate which helps in inhibiting the phago-lysosome fusion [14] and this act as an important marker for the virulent strains. So the structural design of the cell envelope aids in intracellular survival, invasion and other immune modulatory activities [15].

***Mycobacteria* innate resistance to antibiotics:**

Bacterial cell wall is prone to drugs such as penicillin interacts with the β-lactam ring in the peptidoglycan layer and inhibits cell wall synthesis; vancomycin alters the molecular structure of the cell wall. Mycobacterial cell wall containing thick mycolic acid layer with the cell wall containing glycoproteins play a crucial role in resistance towards susceptible drugs [16]. The impermeable nature of the cell wall proves itself to be an inherent property for resistance. Mycobacteria in combination with impermeability utilizes efflux pumps, antibiotic modifying or degrading enzymes or target modifying enzymes to survive even after antibiotic treatment [13].

How TB transmits?

TB is an airborne disease which transmits from an infected person to a susceptible person in the form of airborne particle known as droplet nuclei. These droplet nuclei are tiny water droplets containing the bacilli which are exhaled out in the environment when infected individual cough or sneeze.

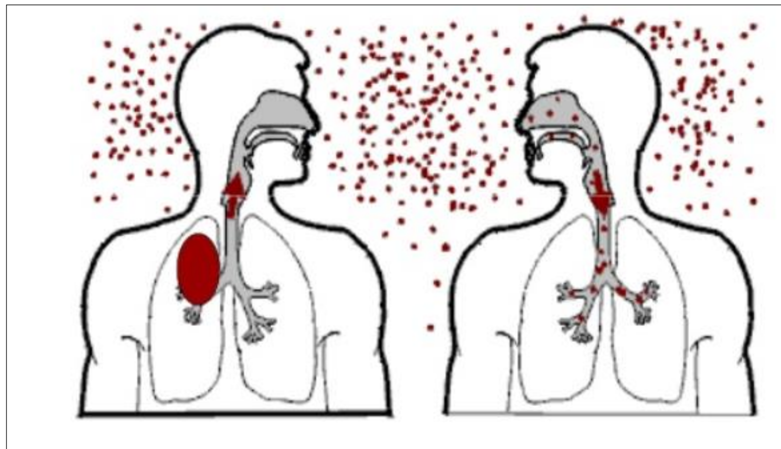


Figure 4

TB survival inside the host:

1. **Onset (1-7 days):** The bacilli is inhaled

2. **Symbiosis (7-21 days):** If the bacilli are not killed by the host immune cells then it reproduces.

3. **Initial caseous necrosis (14-21 days):** Mtb resides in the macrophages and replicates, followed by infecting the other surrounding activated macrophages via oxidative burst.

4. **Interplay of tissue damaging and macrophages activating the immune response (21 days):** Macrophages surround the tubercule but some may be inactive. The

tubercule can break off and spread into the surroundings. If the tubercule spreads into the blood outside the lung then it is called Miliary TB.

5. **Liquefaction and cavity formation:** the tubercule at one point of time will liquefy and will spread even faster. However, very small percentage of the infected hosts attain this stage.

How *Mycobacteria* enters into the host:

The infection is initiated with the phagocytosis of the bacilli into the macrophage upon recognition of the pathogen-associated molecular patterns (PAMP) by specific pathogen recognition patterns (PRPs). PRPs are present in cell surface of Mtb which includes Toll like and mannose receptors and other receptors like lipoproteins and glycoproteins (as mentioned earlier). It also includes lipomannan and mannose-capped lipomannan (ManLAM). These cell surface receptor commence inflammatory response useful for the host, however Mtb can modulate the TLR and MR signaling pathway [17]. This result in the decreased efficiency of antigen presentation of MHC-II by the macrophages [18], restriction of the phagolysosome maturation [19] which subsequently results in inefficiency in T cells activation and thus the *M.tb* evades the host immune response.

The main participating receptors are:

- **TLR:** There are certain lipopeptides and liposaccharides that are TLR ligands, present on the mycobacterial cell wall processes. Interaction of TLR-2 with these ligands leads to the inhibition of the cytokine response [20]. Even prolonged stimulation of TLR-2 leads to the loss in the peptide load in MHC-II, thus though there have been reports regarding protective role of TLR-2 however some reports do contradict with this point [21]. The interaction of M. tb ligands with the thr TLRs sooner or later results in the activation of the NF-κB and in the production of proinflammatory cytokines such as TNF-α, IL-1, IL-12 chemokines and nitric oxide [22].
- **NOD-like receptors**: Nucleotide oligomerization domain are leucine rich protein which interacts with the PAMPs in the cytosol. TNF-α production after mycobacterial infection depends upon these NOD factors. NOD deficit mice showed impaired TNF-α production and nitric oxide synthesis [23].

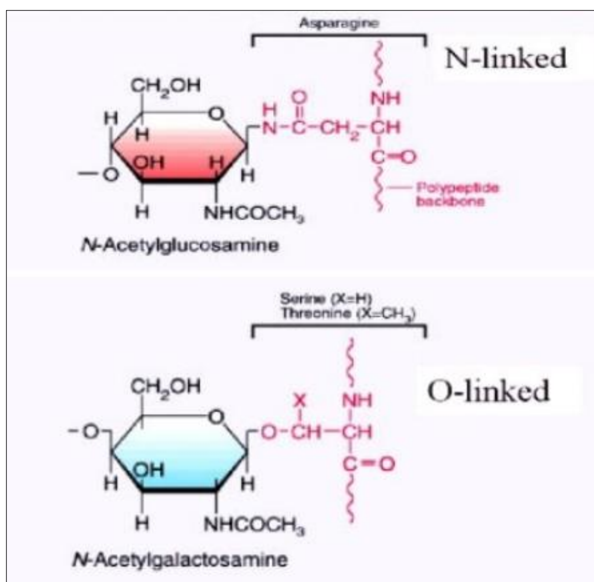
- **Mannose binding receptor**: Mannose receptor, Type 1 transmembrane receptor, dependant phagocytosis takes place after the receptor recognizes the mannose residue present on the cell surface of mycobacteria. Though it's not a professional phagocytosis receptor but is required for the interaction of pathogen with the macrophages and dendritic cells [24].

Mtb glycoproteins:

Glycoproteins are the macromolecules composed of a peptide chain covalently bonded to one or more carbohydrate moieties.

The saccharide chains are referred to as glycans. There are two classes of Glycoproteins

- **O-linked glycans** containing an N-acetylglucosamine attached through a glycosidic bond to O-terminus of either Threonine or serine.
- **N-linked glycans** containing an N-acetylglucosamine attached through a glycosidic bond to N-terminus of an Asparagine residue.



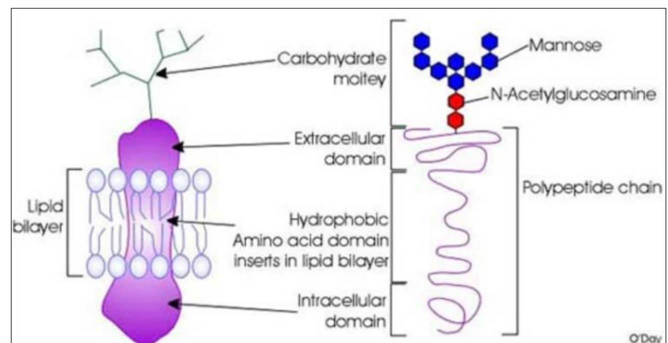


Figure 5

They are located in the extracellular environment or expressed at the cell surface and responsible for various cell to cell interactions [24]. Immunoglobulins, different hormones and surface receptors are some biologically important Glycoproteins.

Glycoproteins are responsible for various infections and parasitic processes [25]. Some pathogens containing glycoproteins that are immune-dominant antigens and can be used for immune-diagnosis and vaccination.

Oxidative stress:

It has been reported that Mtb glycoproteins modulates the redox balance to aid Mtb persistence inside the host macrophages (Mohanty et al., 2015, 2016; Sengupta et al., 2017; Sonawane, Mohanty, Jagannathan, Bekolay, & Banerjee, 2012). Oxidative stress is defined as the imbalance between the production of reactive oxygen species (ROS) and antioxidant defenses. When foreign substances are recognized by the TLRs or MRs and other receptors of innate immunity, during phagocytosis, ROS and RNS are produced. Oxidative stress could results from the following: (1) the presence of xenobiotics, (2) the activation of the immune system in response to invading microorganisms (inflammation), and (3) radiation, which makes oxidative stress a common denominator of toxicity or stress.

How M. tb combats oxidative stress inside the host?

Due to the presence of functional analog of glutathione that is cysteine glycoconjugate, mycothiol and also the presence of superoxide dismutase (SOD), catalase/peroxidase (KatG), thioredoxin reductase (Tpx), alkylhydroperoxide reductase (AhpC) and peroxiredoxin (AhpE) M. tb protects itself from the cellular oxidative stress [26]. And it has been also observed that in case of Gram-negative organisms the OxyR protein plays an important role in the survival against oxidative stresses [27]. Saprophytic *Mycobacterium smegmatis* induced a protective oxidative stress re- sponse analogous to the OxyR response of Gram-negative bacteria [28].

Role of Mitochondria in the production of cellular ROS compounds:

Mitochondria are the main source of cellular ROS compounds inside mammalian cells. Hydrogen peroxide (H_2O_2) one of the commonly known cellular ROS compound forms from the dismutation of superoxide generated within mitochondria. Electron transport chain commonly known as ETC cycle is the main source for the ROS compounds inside the human body. Pathogenic mycobacteria combats survives against this ROS ans RNS compounds due the presence of superoxide dismutase (SOD) and catalase inside it where these ROS compounds (i.e Hydrogen peroxide) is converted to oxygen and water molecules [25]. Whereas non-pathogenic mycobacteria are unable to combat against these ROS and RNS compounds due to the absence of these enzymes.

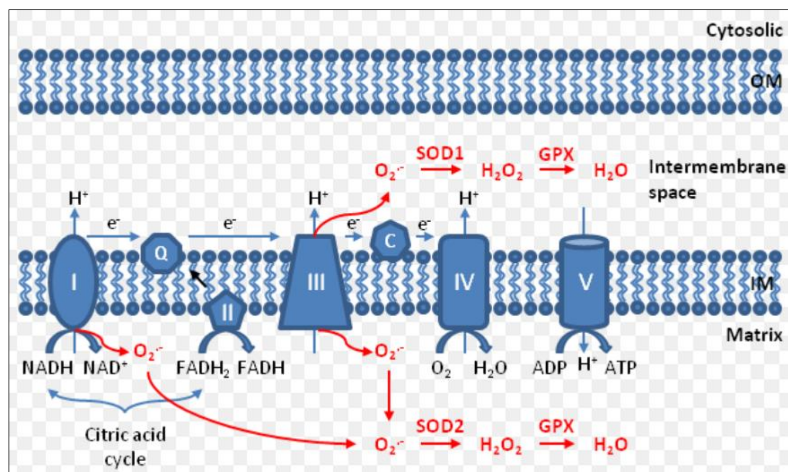


Figure 6

Role of Peroxisomes in regulation of ROS compounds:

Besides mitochondria peroxisomes are also reported to be involved in formation as well as scavenging of ROS. Mammalian peroxisomes play impotant roles in cellular metabolism which includes α and β- oxidation of the fatty acids. The single membrane organelles uses a slightly modified oxidation process to shorten Very long chain fatty acid (VLCFA) to shorter fatty acids that can then be transported to the mitochondria to complete oxidation as mentioned in fig 7. During this metabolism hydrogen peroxide is generated inside the cell which is then further degraded to oxygen and water molecules by catalase present inside the peroxisomes.

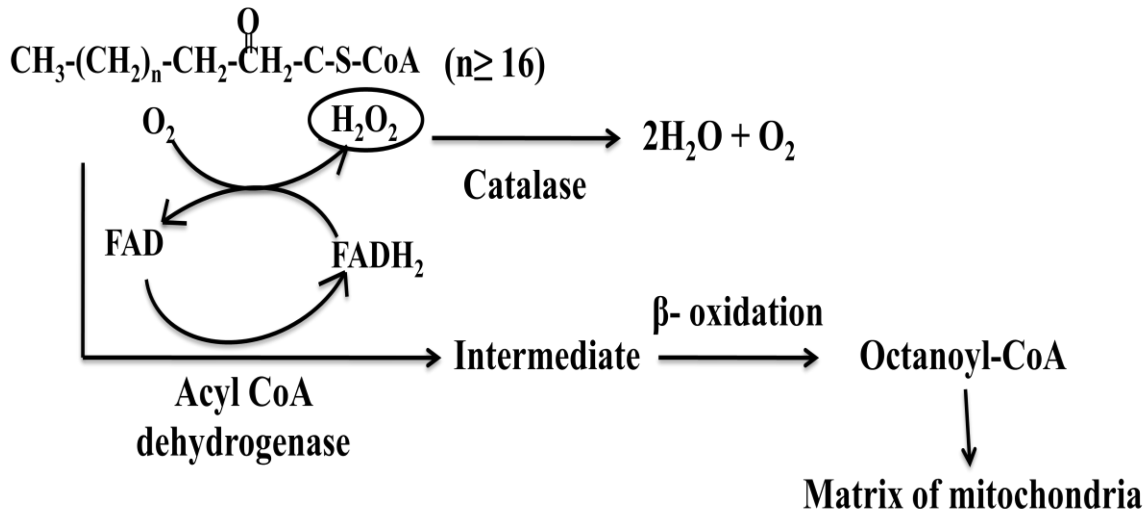


Figure 7

What are Peroxisomes and their functions:

Peroxisomes are simplest sub-cellular organelles that are characteristics of all eukaryotic cells and contain 60 known enzymes in the matrix which also includes 45 integral or peripheral membrane proteins. Peroxisome play some important role in our body mainly in our metabolism those include-

1. Enzymes involved in degradative oxidation such as β-oxidation of very long chain fatty acids.
2. The early steps in the synthesis of ether glycerolipids or plasmalogens.
3. The formation of bile acids, dolichol and cholesterols.
4. The catabolism of purines, polyamines, and amino acids, and the detoxification of reactive oxygen species such as hydrogen peroxide, superoxide anions, and epoxides.

Function of peroxisome and mitochondria in the maintenance of cellular homeostasis:

Mitochondria mainly ETC cycle which is the main source for the cellular ROS compounds, peroxisomes also plays a role in cellular ROS production. Peroxisomes also play an important role in scavenging this cellular ROS compounds. Pexophagy which is commonly known as

degradation of peroxisomal compartments through the formation of autophagosome is basically helps in the maintenance of peroxisomal homeostasis and also helps in peroxisomal biogenesis in mammals [29]. Pex13 peroxin which is mainly found in the peroxisomal membrane helps in selective autophagic degradation of mitochondria that is mitophagy and also of Sindbis virus that is virophagy [31].

What is Autophagy?

The term 'Autophagy' derived from the Greek meaning 'eating of self'. Autophagy is a lysosomal degradative process that plays essential functions in innate immunity, particularly, in the clearance of intracellular bacteria such as *Mycobacterium tuberculosis* and also in the maintenance of cellular homeostasis. Autophagy can be of three different types-

(1) Macroautophagy or classical autophagy, (2) Microautophagy, (3) Chaperon-mediated autophagy.

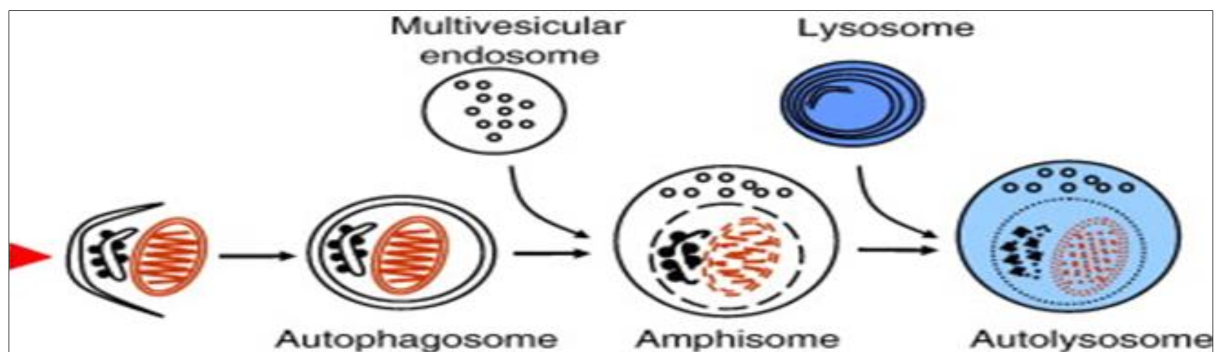


Figure 8

Autophagy pathway and formation of Autophagosome:

It mainly initiates during the time of nutrient depletion. The nucleation of an isolation membrane is the first step for the pathway which is subsequently elongates to envelop the portion of cytoplasm to be removed. During autophagy AMPK are the first molecules to respond which down-regulates the expression of mTORC1, key regulator in autophagic

pathway. Inhibition of mTORC1 further down-regulates the expression of ULK1 which then further inhibit its interaction with Beclin-1 and Atg14L, downregulation of the interaction results in inhibition of autophagic pathway.

Inside the phagophore the LC3-I gets converted to LC3-II and Atg genes such as Atg16L, Atg 12 and Atg5 interacts with the phagophore and forms Autophagosome which then further interacts with the lysosomal compartments to form Autolysosome. Inside the autolysosome compartment the lysosome degrades damaged and unused protein and cytoplasmic organelles.

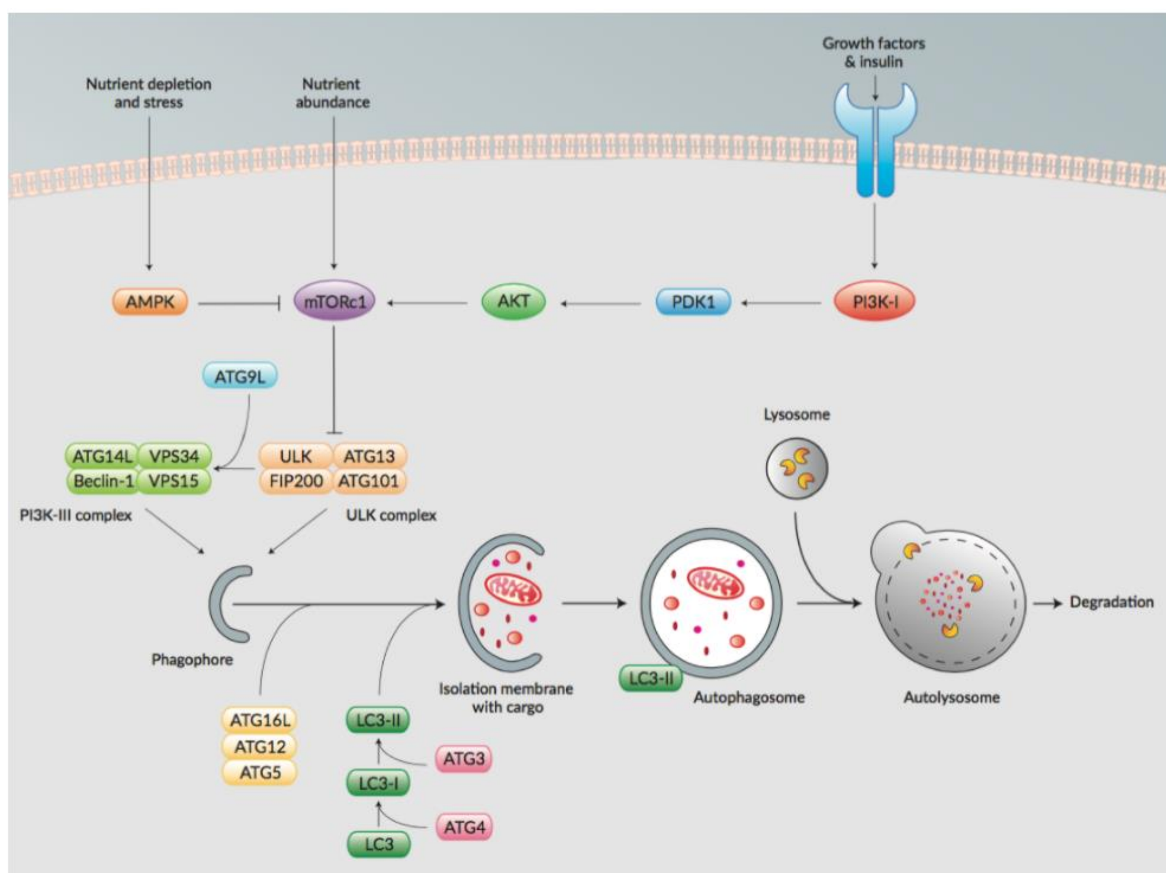


Figure 9

CHAPTER 4

AIMS AND OBJECTIVES

AIM:

The study aims to determine the immunomodulatory role of the *M.tb* gene ACTase in intracellular bacillary persistence by peroxisome dependent inhibition of cellular ROS and autophagy.

OBJECTIVES:

- To construct a recombinant strain of *M.tb* gene ACTase in non-pathogenic *M. smegmatis* using a shuttle vector pSMT3.
- To study the growth kinetics and survival of the recombinant MsmACTase inside macrophage cells.
- To study the effect of MsmACTase in cellular ROS production and its determine its affect on bacillary survival
- To study the effect of MsmACTase in autophagy pathway
- To determine the role of peroxisomes in modulation of ROS and autophagy pathway in MsmACTase infected macrophage cells.

CHAPTER 5

METHODS

BACTERIAL AND CELL CULTURE GROWTH CONDITIONS:

- **a)** *Mycobacterium smegmatis* mc^2155 were grown in Middlebrook's 7H9 broth medium (Difco) containing 0.05% Tween 80, 0.2% glycerol at 37°C on a shaker at 150 r.p.m.
- **b)** *E. coli* XL-10 Gold were grown in Luria-Bertani broth or LB agar supplemented with 20 µg/ml of Tetracycline.
- **c)** **Vector:** pSMT3 plasmid.
- **d)** **Cell lines:** Mouse macrophage RAW 264.7 was maintained in DMEM media (Gibco) supplemented with 10% fetal bovine serum (FBS), 1% antibiotic solution.

METHODS:

1) CLONING OF Mtb ACTase IN Msm:

a) PCR amplification:

PCR amplification of Mycobacterium tuberculosis ACTase, ACTase orthologue in *M.smegmatis* (MSM_2335) genes were carried out by using *M.tb* genomic DNA and *M.sm* genomic DNA as a template using gene specific primers (Table 1) respectively. The PCR conditions are given in (Table 2) and (Figure 9). The PCR product was then digested with *PstI* and *HindiIII* (Table 3) subsequently ligated (Table 4) with pSMT3 vector.

TABLE 1: Primers used

Gene Name	Sequence
ACTase forward	5'-GGA TCC GTG AAC GTC CTC AGT TTG GGC TCG T-3'
ACTase reverse	5'-AAG CTT CTA GCG GGC CGC CTT CTT GC-3'

TABLE 2: The PCR mixture includes

Reaction components	**Volume (µl)**
MQ water	21.2
10X Paq Buffer	3.5
dNTP mix	3
Template (Genomic DNA)	3
Forward primer(refer Table 2)	2
Reverse primer(refer Table 2)	2
Paq polymerases	3

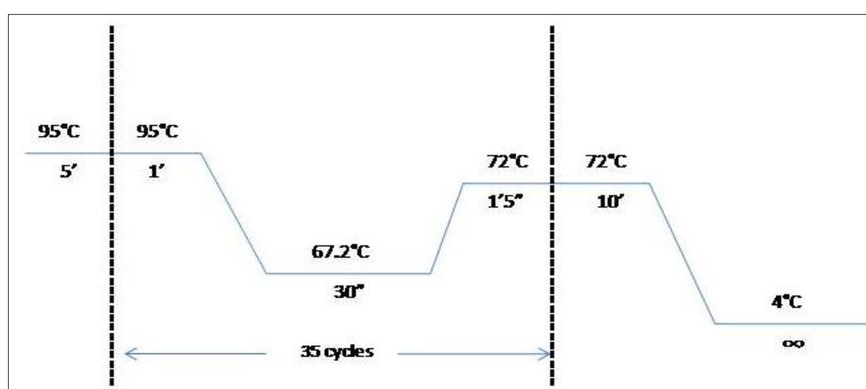


Figure 10: PCR conditions

TABLE 3: Parameters for Restriction digestion includes

Reaction mixture components	**For plasmid (µl)**	**For insert (µl)**
MQ water	15.9	15.9
Buffer 10X	3.5	3.5
pSMT3 plasmid	15	------
Insert	-----	15
HindIII	0.3	0.3
PstI	0.3	0.3

TABLE 4: Ligation mixture includes

Reaction Components	Test Sample (µl)	Control (µl)
MQ water	10	19
T4 DNA Ligase Buffer (10X)	2.5	2.5
Plasmid	3	3
Insert	9	---
T4 DNA Ligase	0.5	0.5

b) Preparation of competent cells:

E. coli XL-10 gold overnight grown culture was subcultured into 100ml LB broth and kept in shaking incubator for at least 3 hrs (O.D 0.6-0.7). After 3 hrs the growth was blocked by keeping the flask in ice for 30minutes. The culture were then equally divided into 50 ml falcon tubes and centrifuged at 3500 r.p.m for 10 minutes in a precooled centrifuge at 4°C. The supernatant was discarded and the pellet was resuspended in 80mM and 50mM $CaCl_2$ and $MgCl_2$ respectively. The tubes were then again centrifuged at 3500 r.p.m for 10 minutes at 4°C. The supernatant was discarded and pellet was dissolved in 0.1 M $CaCl_2$ and 50% glycerol. Aliquots of 80µl were prepared and used or stored as per requirement.

c) Transformation:

About 5-7µl of the ligated product was added to 80µl of the previously prepared competent *E. coli* XL-10 gold cells. The aliquots were then incubated for 15-20 minutes in ice. The water bath was pre set to 42^0C. After the 15-20 minutes incubation in ice the tubes were then subjected to heat shock for 90 sec at 42^0C. Just after 90sec the tubes were immediately transferred to ice for 20 minutes. This immediate transfer should be done carefully and quickly as because the pores that are created during the heat shock for the entry of DNA are closed during the ice incubation. 400 µl of LB media were added to the tubes and the tubes were incubated at 37^0C for about an hour. Post incubation the tubes were centrifuged at 1000 r.p.m for 5minutes and the pellet was resuspended in 200 µl of LB media. The tubes were then plated in LB-hygromycin media by spread plate technique and incubated at 37^0C overnight.

d) **Screening of positive colonies:**

Several transformed colonies in overnight incubated plates were restreaked in hygromycin (100µg/ml) containing LB plates. For confirmation of positive colonies, few colonies were selected and colony PCR was performed with the gene specific primers (Table 1). The amplification was observed by running the samples in gel electrophoresis. For further confirmation they were double digested with *HindIII* and *Pst I* to check for the insert release.

Transformation of the positive colonies into the electrocompetent cell:

Preparation for electrocompetent cells:

To prepare electrocompetent cells, *M.smeg* was inoculated in 5ml LB culture flask and incubated for 2days at 37^0C. After 2days it was subcultured to 100ml flask containing LB broth. The growth was arrested at O.D 0.6-0.7 and kept in ice for about 20-30 minutes. The culture were then equally divided into two 50 ml falcon tubes and centrifuged at 8000 r.p.m for 10 minutes in a precooled centrifuge at 4°C. The media was discarded carefully and the pellet was resuspended in 35 ml of chilled MQ and incubated in ice for 20 minutes. The tubes were then again centrifuged at 8000 r.p.m for 10 minutes at 4°C. The pellets were again resuspended in 15ml of chilled MQ and kept in ice for 20 minutes and again centrifuged at 8000 r.p.m for 20 minutes at 4°C. The pellets were resuspended in 5ml of child 10% glycerol and kept in ice for 20 minutes, then centrifuged at 8000 r.p.m for 10 minutes at 4°C. The glycerol was discarded and pellets were resuspended in 500µl 10% glycerol and aliquots of 60-80 µl were made and used for transformation or was stored at -80°C.

Electroporation:

About 4 µl of the insert was added to the 80 µl electrocompetent cell and mixed gently. The content was transferred to prechilled electroporation cuvettes and incubated in ice for 10minutes. The electroporation was carried out at 2500V/5 millisec. Then the content was transferred to a prewarmed (42^0C) 1ml LB broth. The tubes were then left at RT for 5-10 minutes. The tubes were incubated for 50 minutes at 37^0C in shaking incubator (150-200 r.p.m). 10-50µl of the content was plated.

2) ANALYZING THE GRWOTH KINETICS:

To analyze the growth patterns of *M.smeg* (WT), pSMT3 and recombinant *MsmACTase,* they were cultured in 100ml of LB broth containing 0.5% Tween80 and hygromycin (50 µg/ml, for recombinant strains) by transferring 0.1% of fresh inocula (O.D= 0.1) into it. The flasks were incubated at 37^0C with 120rpm. The growth kinetics was assayed by measuring the O.D at 600nm every after 12 hrs.

3) INTRACELLULAR SURVIVAL ASSAY:

Mouse macrophages (RAW264.7) cells ($2x10^5$ cells/well) were seeded on 24-well tissue culture plates and grown for 18-20 h. Cells were infected with pSMT3 and MsmACTase strains at a MOI 10 for 2 h. After 2 h of infection, the cells were washed with 1XPBS 3 times to remove the extracellular bacteria. After 6 and 24 h of infection cells were again washed with 1X PBS, lysed with 0.5% Triton-X-100 and intracellular bacterial count (0h) was estimated by plating serially diluted samples on 7H9 plates. Plates were incubated at 37 ^{0}C and *Msm* colonies were enumerated after 3 days. The survival percentage was calculated with respect to the number of bacteria present in the inoculum termed as "pre-inoculum density (PID)" used to infect the cells. The PID of each bacterial strain was considered as 100% and the CFU counts obtained after the cell infection was used to calculate the percentage of intracellular survival of bacteria.

4) FLOW CYTOMETRIC ANALYSIS:

RAW264.7 ($4.5x10^5$ cells/well) were seeded onto 6-well plates and infected with pSMT3 and MsmACTase at an MOI 10 for 24 h. Total ROS and superoxide anions (O_2-) generated were measured by using 2', 7'-dichlorofluorescein diacetate (DCFH-DA) and dihydroethidium (DHE) dyes, respectively. Then cells were harvested in 1X PBS containing 0.1mg/ml DNase (Himedia, Mumbai) and analyzed using Flow cytometer (BD FACS Canto II cytometer) and analyzed using FACS Diva software. Forward scatter (FSC) area vs height measurement was done to remove clumps for single cell analysis and single cells falling

along a diagonal were chosen for further analysis. Forward scatter (FSC) and side scatter (SSC) were used to gate viable and single cell events. Appropriate gating was done to exclude debris and dead cells from analysis by categorizing low-FSC events as debris, and low FSC and high SSC as dead cells. A compact cell population was thus gated based on size and granularity. The gated cells were further analyzed for uptake of impermeable propidium iodide (PI) (Invitrogen) stain to determine live versus dead cells. Gated FITC positive cells from stained and unstained controls, pSMT3 and MsmACTase were over-layed to determine the shift in the population using FlowJo (USA) for invasion assay.

5) WESTERN BLOT ANALYSIS:

Expression profiling of autophagy markers and transcription factors was checked by Western blotting technique after infection with pSMT3 and MsmACTase at a MOI 10 on RAW264.7 cells. The infection assay was same as followed for the other experiments performed above. 24hrs post infection, cells were harvested by using lysis buffer and cells were stored at -80°C overnight. The lysate was then centrifuged at 13,000 rpm for 25 mins at 4°C and proteins were electrophoresed by using SDS-PAGE and the electrophoresed protein was then transferred to PVDF membrane. After western blotting the membrane was blocked by using 5% skim milk or 5% BSA in PBST for 2-3 hrs and then left for overnight incubation by using primary antibody in PBST. Next day the blot was washed using PBST 3 times for 5 minutes each. After washing blot was again incubated by using secondary antibody in PBST for 2-3 hrs and again washed for 3 times as before. After washing was done X–ray film was developed using standard chemiluminiscent solvent.

Expression profiling of autophagy markers and transcription factors was checked by Western blotting technique after treating the cells with Zymosan, ros inducer [32] at a concentration of 200µg/ml for 2 hrs followed by infection procedure as described above.

6) IMMUNO-FLUORESCENCE MICROSCOPIC ANALYSIS:

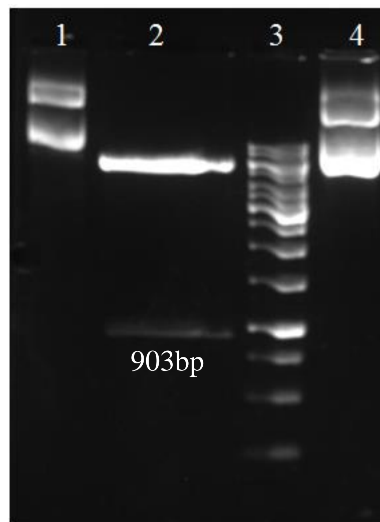
RAW 264.7 cells were seeded on coverslips in 24 well plates followed by infection with WT, pSMT3 and MsmACTase for 24 hrs. After 24 hrs media the cells were washed twice with 1X PBS and fixed with acetone/methanol (1:1) and kept at -20°C for 20 mins. After 20 mins of

incubation cells were again washed with 1X PBS for 3 times and blocked in 200µl of 1% BSA/0.5% saponin solution and kept at 37°C for 1 hr. Then 1 hr post blocking the coverslips with cells were again washed with 1X PBS for 5-7 times and placed on a box with parafilm containing primary antibody (1:200) and kept it overnight at 4°C. Next day the coverslips were again washed with 1X PBS for 5-7 times and Alexa-Flour tagged 2° Antibody (1:200) was added and kept for another 2 hrs at 37°C in dark. After 2 hrs of incubation coverslips were again washed and mounted on slides with 2µl of mounting solution containing DAPI and left to dry.

CHAPTER 6

RESULTS

4.1 <u>CLONING:</u>



Lane 1: Undigested positive colony
Lane 2: Digested positive colony
Lane 3: 1kb ladder
Lane 4: Undigested pSMT3 plasmid

Figure 11: Gel image showing the cloned ACTase in *Msm*

PCR amplification of ACTase was carried out using gene specific primers and run on a 1% agarose gel. As anticipated the amplified bands were observed at 903bp in the gel electrophoresis. The band which showed more intensity was eluted and digested with *HindIII* and *PstI*. The digested product was ligated subsequently with pSMT3 vector and further transformed into *E.coli* XL 10 Gold competent cells. The positive colonies were then transformed into electrocompetent cells of *M.smegmatis*, and confirmed by double digestion and checked for the insert release.

4.2 IN VITRO GROWTH KINETICS:

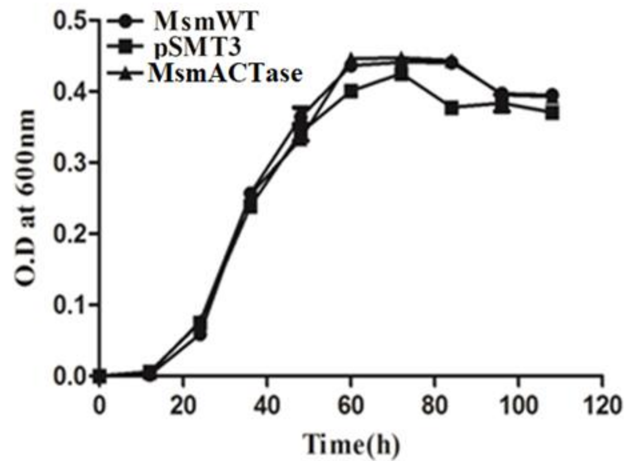


Figure 12: Graph showing the growth pattern of MsmWT, pSMT3 and MsmACTase

The growth kinetics of the cloned constructs WT, pSMT3 (vector control), MsmACTase were analysed by inoculating and subculturing them in 100ml of 7H9 media and hygromycin (50µg/ml, for recombinant strains), for specialized time period (0-108hrs). It was observed that no considerable differences were observed in the growth rates of the recombinant strain from the WT and vector control (from the compared O.D at 600nm), suggesting that over-expression of this gene does not lead to growth defects in vitro. This indicates that the observed phenotypes would be due to the gene expression, not because of growth defects.

4.3 Recombinant MsmACTase results in increased survivability inside phagocytic cells:

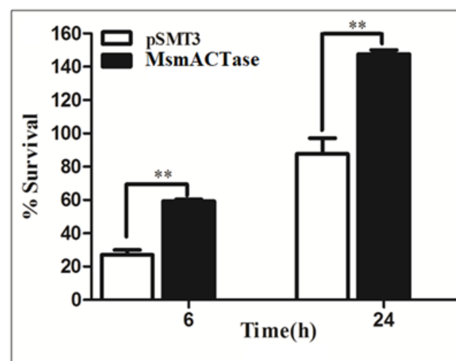


Figure 13: Intracellular survival of pSMT3 and MsmACTase by CFU assay

Survival assay analysis was done to check the intracellular bacterial count in phagocytic cells at time points 6 and 24 h post infection. Mouse macrophage RAW 264.7 cells were infected with pSMT3 and MsmACTase and the survival of the strains were checked by CFU assay. It was observed at all time points MsmACTase showed more survival as compared to pSMT3. This indicated that MsmACTase is able to survive inside macrophages.

4.4 MsmACTase down-regulates ROS for its persistence inside the macrophages:

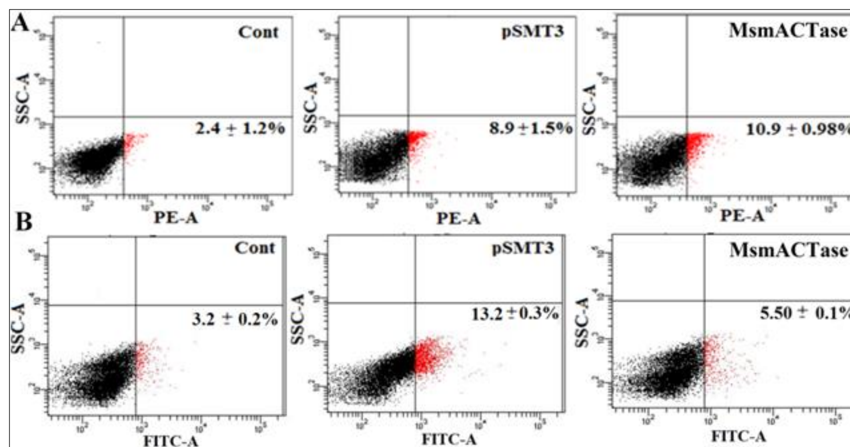


Figure 14: ROS production was determined by DHE (A) and DCFH-DA (B) staining

Redox balance is crucial for the intracellular survival of mycobacteria [33]. *Mtb* is known to inhibit host oxidative stress responses to facilitate its survival in macrophages. To determine the rationale behind the increased intracellular persistence of MsmACTase we therefore, we first measured the level of ROS production in pSMT3 and MsmACTase infected RAW264.7 cells using DHE and DCFH-DA staining. DHE staining did not show any significant difference in the production of superoxide anions (O_2^-), precursor for ROS and NO production, in *MsmACTase* (10.9±0.98%) and *Msm* pSMT3 (8.9±1.5 %) infected macrophages (upper panel, Fig 14A). However, DCFH staining showed significantly less ROS production in *MsmACTase* (5.5±0.1%) as compared to *Msm* pSMT3 (13.2±0.3%) infected macrophages (middle panel, Fig 14B). This indicated that MsmACTase inhibits ROS to survive inside the macrophages.

4.5 Msm ACTase down-regulates autophagy for its survival inside host:

Autophagy is a cellular defense mechanism against pathogenic microorganisms upon infection. Autophagy was checked by using classical autophagic which are mainly involved in the regulation of cellular autophagy including mTORC1, Atg5, Atg 7, Beclin-1, p-ULK1, ATM-kinase, p-AMPK and LC3-II which mainly helps in the formation of autophagosomes.

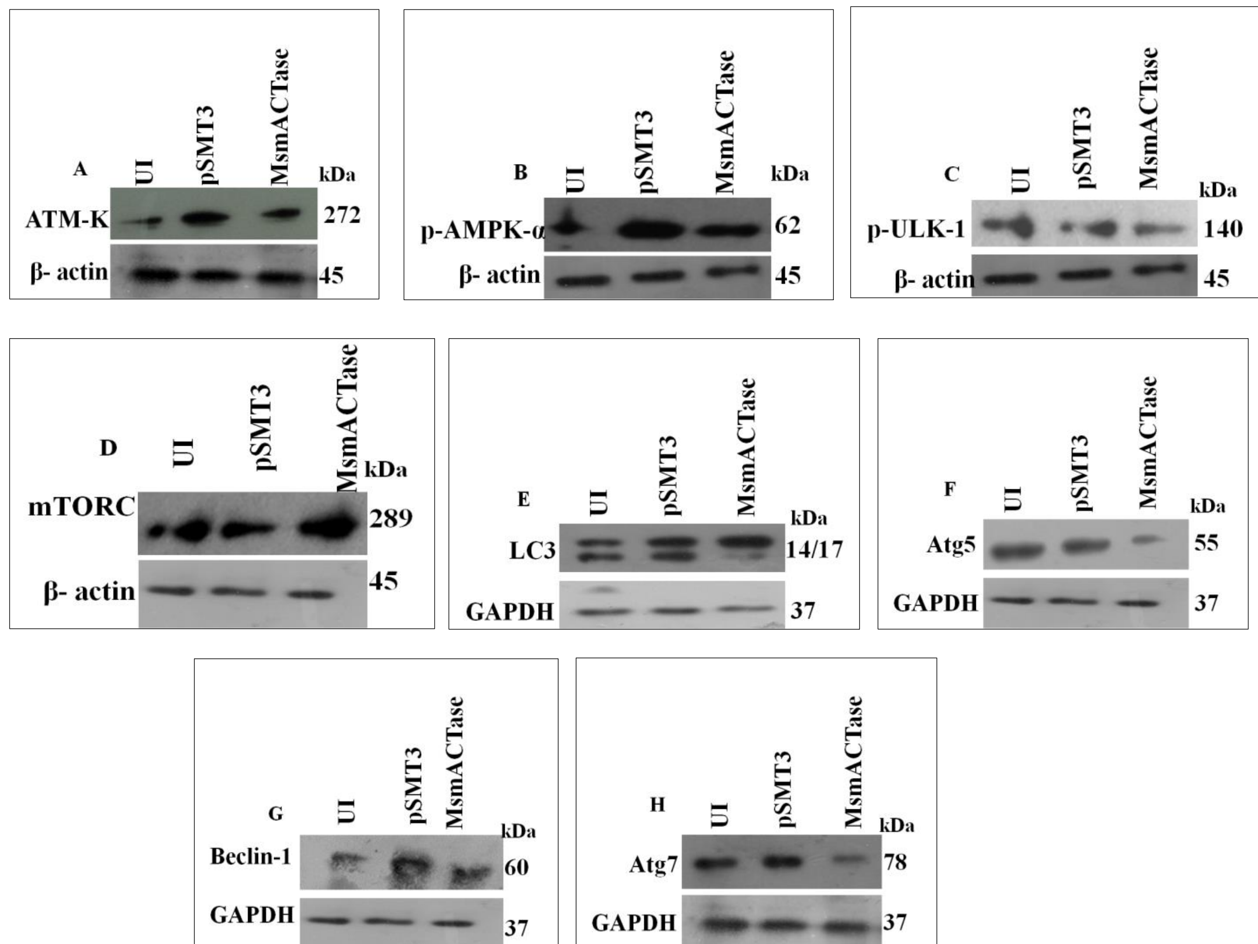


Figure 15: Western blot analysis showed that MsmACTase infected cells downregulation of autophagy markers (A) p-ATM kinase (B) p-AMK-α (C) p-ULK-1 (E) LC3II (F) Atg5 (G) Beclin-1 and (H) Atg7. Upregulation of mTORC-1 expression was observed as a result of p-ULK-1 downregulation in MsmACTase infected cells.

As shown in Fig 15 A, the first responder due to ROS dependant DNA damage was downregulated in case of MsmACTase infected cells which indicated that inhibition of ROS might have blocked the autophagy pathway. To further determine the same we checked for the expression of classical autophagy regulating genes [34] like p-AMPKα (Fig 15 B), gene

p-ULK1 (Fig 15 C). Whereas Fig. 15 D MsmACTase infection showed the up-regulation of mTORC-1 expression (known to inhibit autophagy by down-regulating the genes p-ULK1 and p-AMPKα) also signifies that MsmACTase aids in down-regulation of autophagy in host macrophages. LC3 is an essential autophagy regulating gene helps in the formation of autophagosome which upon up-regulation of autophagy converts to LC3II here in Fig 15 E it was clearly observed that there was a down-regulation of LC3II expression in MsmACTase when compared to vector control. Other autophagy related genes like Atg5, Atg7 and Beclin-1 (Fig. 15 F-H) also showed down-regulation in case of MsmACTase when compared with the uninfected and vector control.

4.6 Fluorescence microscopic analysis of autophagy marker pAMPKα

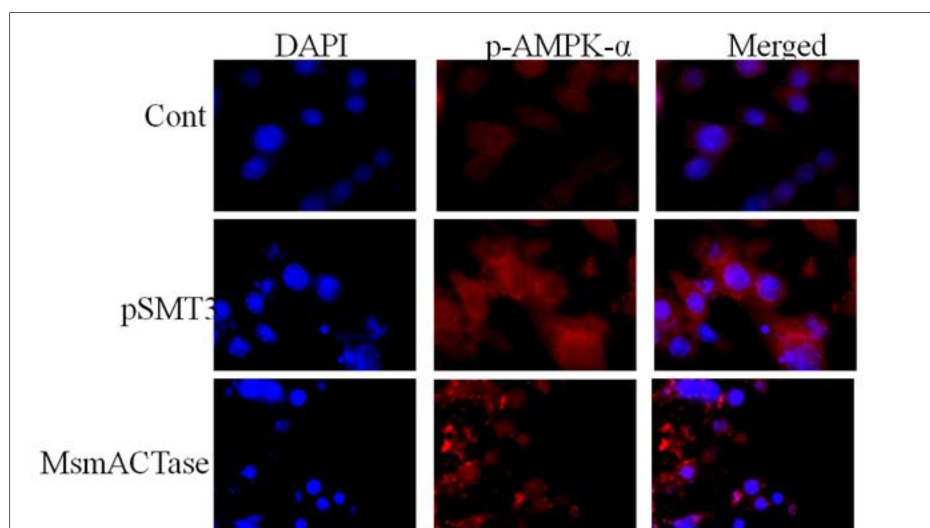


Figure 16: Fluorescence microscopy image showing the expression of pAMPKα in RAW 264.7 pSMT3, MsmACTase and Uninfected cells.

To further validate the western blot result we performed immunostaining assay with p-AMPK-α antibody using fluorescence microscopy. It was observed that the expression of classical autophagy marker pAMPKα significantly decreased in pAMPKα in MsmACTase infected cells when compared with the UI and pSMT3 infected cells. Thus the result further corroborated that MsmACTase down-regulates the expression of autophagy for its persistence inside the host macrophage cells.

4.7 Autophagy is upregulated when ROS is induced in MsmACTase infected cells:

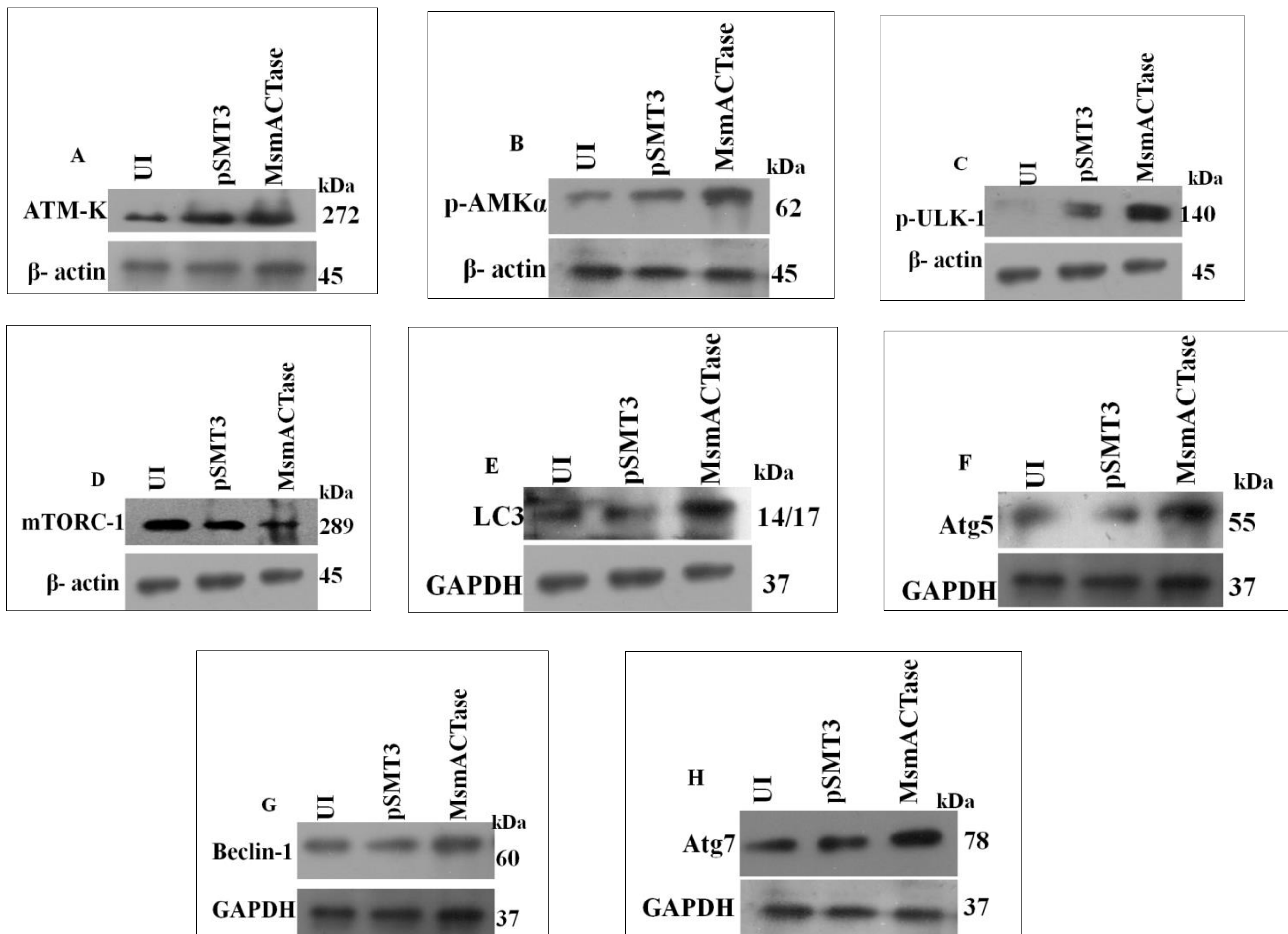


Figure 17: Western blot analysis post Zymosan treatment showed that MsmACTase infected cells upregulated the expression of autophagy markers (A) p-ATM kinase (B) p-AMK-α (C) p-ULK-1 (E) LC3II (F) Atg5 (G) Beclin-1 and (H) Atg7. Downregulation of mTORC-1 expression was observed as a result of p-ULK-1 up-regulation in MsmACTase infected cells.

Upon induction of ROS by Zymosan treatment in cells prior to infection with pSMT3 and MsmACTase showed upregulation of autophagy markers like p-ATMkinase (Fig 17 A), p-AMPKα (Fig 17 B), gene p-ULK1 (Fig 17 C), LC3II (Fig 17 E), Atg5 (Fig 17 F), Beclin-1 (Fig 17 G) and Atg7 (Fig 17 H) in MsmACTase infected cells. Expression of m-TORC-1 was found to be downregulated in MsmACTase infected cells.

4.8 MsmACTase down-regulates the expression adaptor molecules and transcriptional factors which are responsible for cellular autophagy:

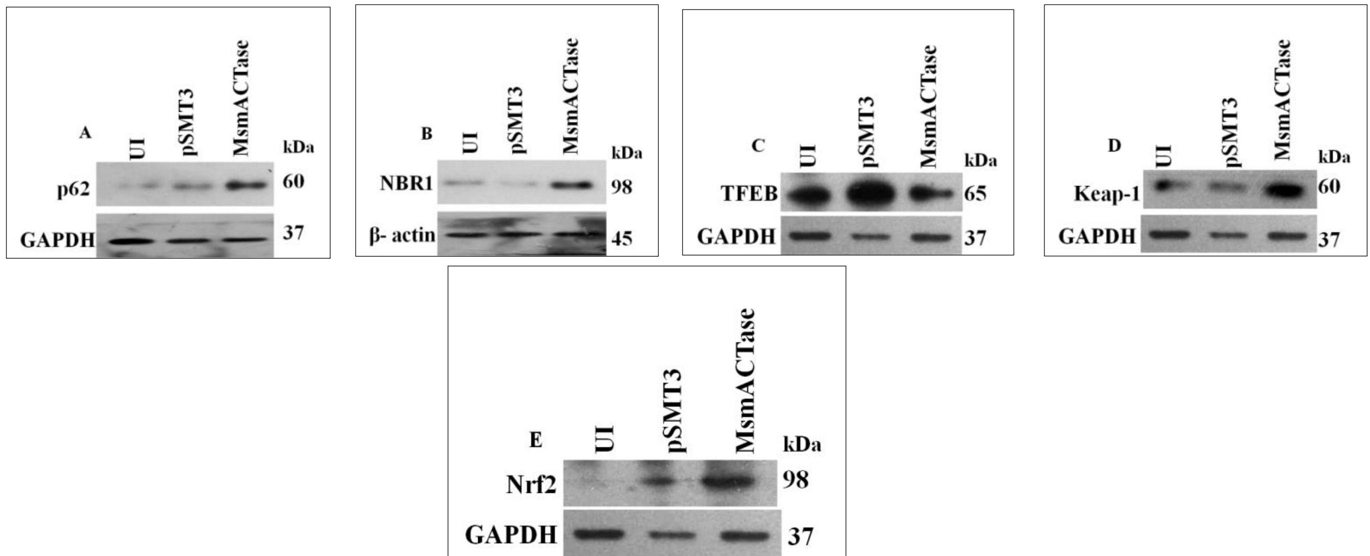


Figure 18: Western blot analysis to check the expression of the autophagy related adaptor molecules (A and B) and transcriptional factors (C-E) 24 h post-infection with pSMT3 and MsmACTase.

p62 and NBR1 are the two autophagy adaptor molecules which play a major role in autophagosome formation. It was observed that 2h of post infection the expression of p62 and NBR1 (Fig 18A and B) is relatively higher in MsmACTase infected cells than the vector control. Further xpression of transcriptional factor TFEB was observed to be downregulated in MsmACTase which further corroborated with the upregulation of mTORC-1 expression (as it has been previously reported that mTORC-1 phosphorylates the serine residue of TFEB thereby inhibiting its translocation to the nucleus). Whereas the other transcription regulating genes expression i.e Keap-1 and Nrf2 also up-regulated when compared with the uninfected (Fig 18D and E).

4.9 Induction of ROS modulated the expression adaptor molecules and transcriptional factors which are responsible for cellular autophagy:

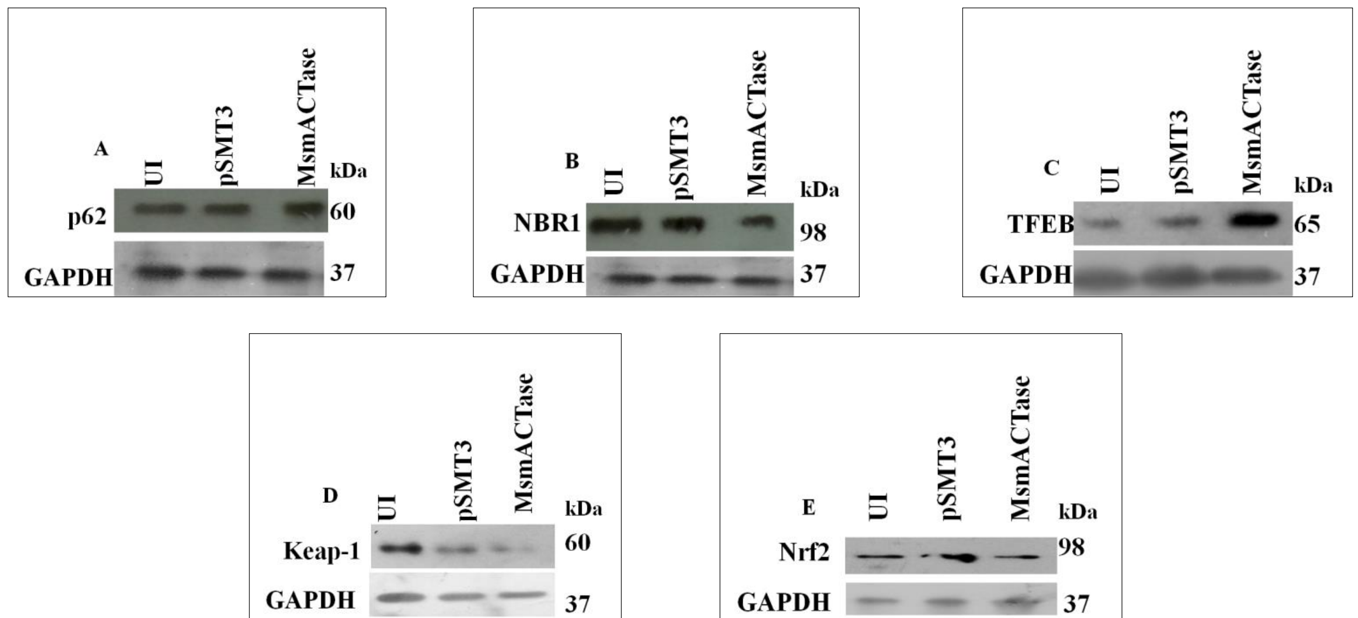


Figure 19: Western blot analysis to check the expression of the autophagy related adaptor molecules (A and B) and transcriptional factors (C-E) 24 h post-infection with pSMT3 and MsmACTase in Zymosan treated RAW 264.7 cells.

Upon induction of cellular ROS the expression of autophagy adaptor molecules like p62 (Fig 19 A) and NBR1 (Fig 19 B) were found to be downregulated in case of MsmACTase infection. This correlated with the higher expression of LC3II expression in Zymosan treated cells. To further confirm we determined the expression of the transcription factor TFEB, which aids in lysosomal biogenesis. We observed that MsmACTase infected cells showed upregulation of TFEB (fig 19 C) thereby validating that the autophagy pathway is getting upregulated. It has been reported earlier that the Nrf2 bound Keap1 system is inactivated when autophagy is upregulated, corroborating to the previous finding we observed that induction of autophagy due to increased ROS downregulated the expression of both Keap1 (Fig 19 D) and Nrf2 (Fig 19 E).

4.10 Inhibition of peroxisomes upregulated ROS production MsmACTase infected cells:

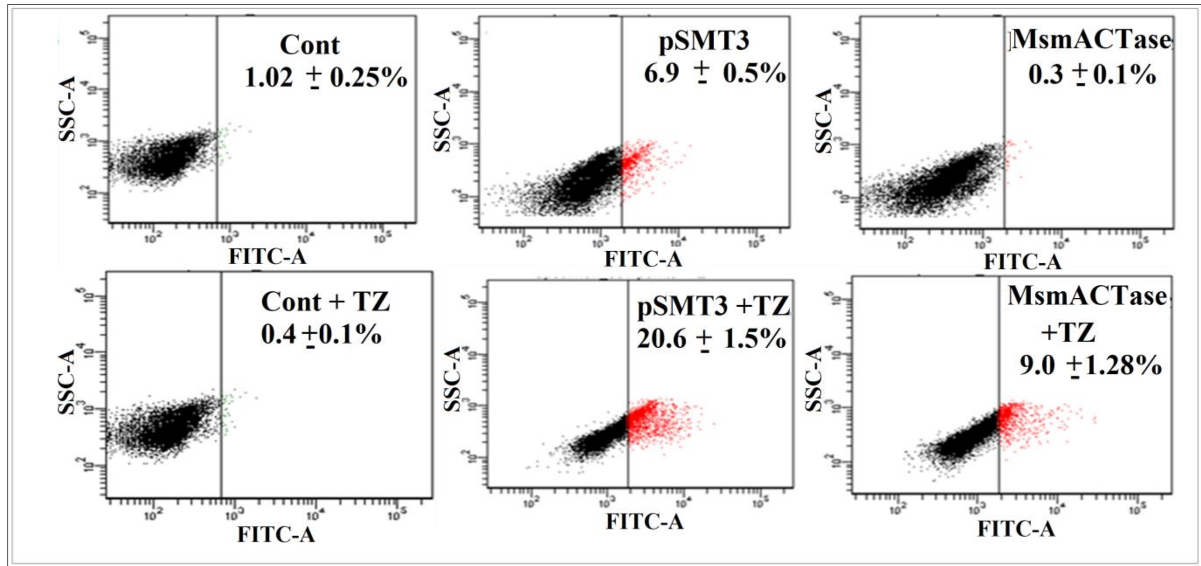


Figure 20: ROS production by DCFH-DA staining in post infection with pSMT3 and MsmACTase in cells treated with (bottom panel) and without peroxisome inhibitors (upper panel)

From the previous reports it has been known that peroxisomes play crucial role in maintaining redox balance, therefore we further determined the role of peroxisomes in ROS modulation in pSMT3 and MsmACTase infected cells. We observed that treatment with peroxisome inhibitor, TZ(10µM) increased the ROS production (9.0±1.28%) in MsmACTase infected cells when compared to untreated conditions (0.3±0.1%). This indicated that peroxisomes are playing a crucial role in regulating the ROS production in MsmACTase infected cells, thereby helping the recombinant strain to survive inside the macrophages.

4.11 Inhibition of peroxisomes upregulated the expression of autophagy related genes and transcriptional factors in MsmACTase infected cells:

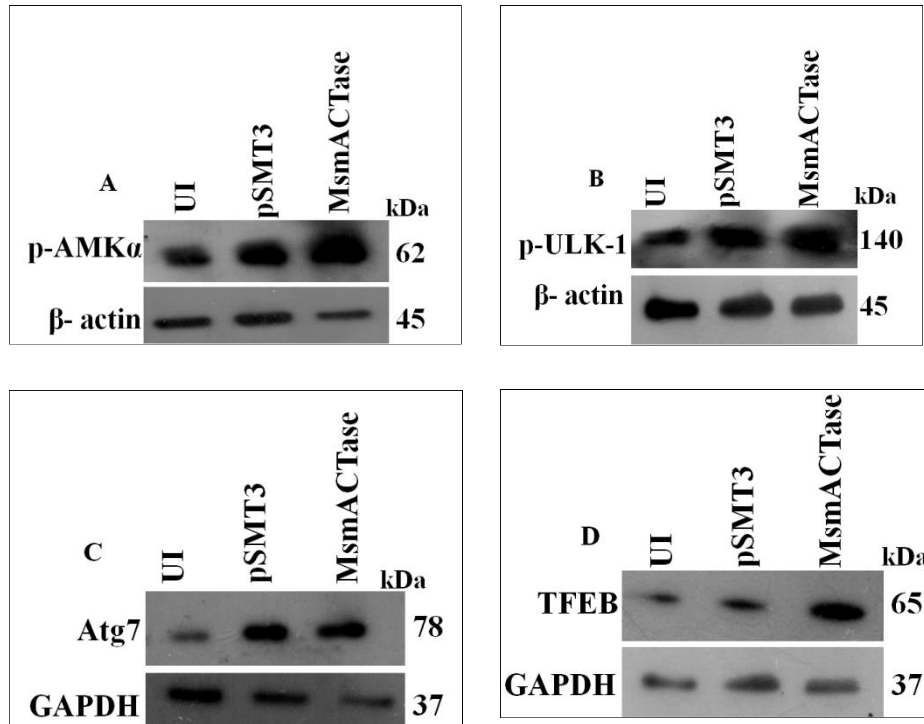


Figure 21: Western Blot analysis to check for the expression of autophagy related genes (A) p-AMPKα, (B) p-ULK-1, (C) Atg7 and (D) TFEB after infection with pSMT3 and MsmACTase in presence of peroxisome inhibitor.

To further confirm the role of peroxisomes in autophagy post infection, we infected the cells with pSMT3 and MsmACTase and then inhibited the functionality using TZ (Thiodiazine hydrochloride, chemical inhibitor for peroxisomal β- fatty acid oxidation). Post treatment it was observed that the expression of representative autophagy related genes were up-regulated in case of MsmACTase infected cells further confirming that MsmACTase inhibits ROS and autophagy pathway in peroxisome dependant manner.

CHAPTER 7

DISCUSSION

M. tb employ various strategies to subvert host immune responses for its persistence in macrophage, one of the component are the *M. tb* glycoproteins are credited to play crucial role in virulence as well as in antigenic processes due to their ability to manipulate host antibacterial effector mechanisms. However, considering the complexity of *M. tb* genome the function of many glycoproteins is still undefined. Several mycobacterial membrane proteins are known to restrict the host immune responses to facilitate the bacterial survival. One of the mechanisms used by the pathogen to weaken the immune response is to subdue the production of oxidative stress molecules in host cells. Although *Mtb* is known to inhibit host oxidative stress mechanisms, the underlying molecular mechanisms are poorly understood. In our study we aim to characterize a functionally unknown *M.tb* gene encoding for acetyltransferase in Msm, which is surrogate lab model of mycobacteria. The gene was ectopically expressed in the non-pathogenic strain using a shuttle vector, pSMT3. MsmACTase, the recombinant strain showed no difference in growth kinectics when compared to WT (*M. smegmatis*) and the vector control. This indicated the phenotype observed would be due to the expression of ACTase and not due to the growth defects. From the intracellular survival CFU counts we observed that MsmACTase aids in the intracellular survival of the bacilli inside the host macrophages. As reported by earlier findings our results also corroborates the role of acetyltransferase in bacterial survival, in which *Mtb* acetyltransferase Eis protein and arylamine N-acetyltransferase were shown to play an important role in intracellular survival of *Mtb* in macrophages [35].

Pathogenic mycobacteria inhibit oxidative burst mechanisms to facilitate its survival inside the host cells. These pathogens either develop resistance mechanisms or employ various detoxification pathways to combat the oxidative stress. ROS especially H_2O_2 exhibits potent toxic effects on the invading pathogens. It can easily diffuse through the cell membrane and generate oxidative radicals [36]. How *Mtb* counteracts this oxidative stress environment in host cells remains elusive. Here we provided sufficient evidences that *MsmACTase* inhibit ROS production in infected macrophages. This is in agreement with a previous report where deletion of *Mtb eis*, encoding for acetyltransferase, resulted in increased ROS production [37]. Macrophages infected with recombinant MsmACTase strain were associated with decreased cellular ROS production, indicating that increased survival seen in macrophages is due to the inhibition of ROS production.

Mitochondria, which plays an important role in the production of cellular ROS also plays crucial role in maintain cellular autophagy. Up-regulation of cellular ROS also up-regulates autophagy, mechanism which plays a crucial role in regulation of mycobacterial growth inside macrophages. Classical autophagy regulating LC3II and other genes such as mTOR, pAMPkα, pULK1 and other transcriptional factors TFEB, Keap-1, p62, NBR1, Nrf2 plays significant role in regulation of autophagy. TFEB helps in the lysosomal biogenesis [38] which further helps in the autolysosomal complex formation, upon up-regulation of mTOR phosphorylates TFEB [39] and down-regulates the lysosomal biogenesis. The autophagy response to MsmACTase was identified by the expressions of the autophagy markers, recombinant MsmACTase found to play major role in the down-regulation of these autohagy genes including the transcriptional factors. The present study provided evidence that MsmACTase which is an essential gene for the survival of M. tb inhibit programmed autophagy mechanism and thus enable the pathogen to increase its persistence inside the macrophages.

CHAPTER 8

CONCLUSION

In conclusion, this work has identified a novel *M.tb* acetyltransferase and demonstrated previously unknown mechanistic insights on how *MsmACTase* interferes with the host immunity and generates a suitable niche for intracellular bacillary persistence by modulating the oxidative stress responses and inhibiting the self degradative autophagic pathway. In the present study functional unknown MsmACTase was shown to down-regulate ROS production inside the cell which aid in increased intracellular persistence. As part of the future study we aim to determine the role of mitochondria, which is the main source of cellular ROS, in MsmACTase persistence inside the macrophages.

REFERENCES:

1. Fransen M, Nordgren M, Wang B, Apanasets O. Role of peroxisomes in ROS/RNS-metabolism: implications for human disease. Biochim Biophys Acta. 2012 Sep;1822(9):1363-73. doi: 10.1016/j.bbadis.2011.12.001. Epub 2011 Dec 9. Review. PubMed PMID: 22178243
2. Ehrt S, Schnappinger D. Mycobacterial survival strategies in the phagosome: defence against host stresses. Cell Microbiol. 2009 Aug;11(8):1170-8. doi: 10.1111/j.1462-5822.2009.01335.x. Epub 2009 May 6. Review. PubMed PMI 19438516; PubMed Central PMCID: PMC3170014
3. MacMicking JD, North RJ, LaCourse R, Mudgett JS, Shah SK, Nathan CF. Identification of nitric oxide synthase as a protective locus against tuberculosis. Proc Natl Acad Sci U S A. 1997 May 13;94(10):5243-8. PubMed PMID:9144222; PubMed Central PMCID: PMC24663
4. Tyagi P, Dharmaraja AT, Bhaskar A, Chakrapani H, Singh A. Mycobacterium tuberculosis has diminished capacity to counteract redox stress induced by elevated levels of endogenous superoxide. Free Radic Biol Med. 2015 Jul;84:344-354. doi: 10.1016/j.freeradbiomed.2015.03.008. Epub 2015 Mar 27.PubMed PMID: 25819161; PubMed Central PMCID: PMC4459714
5. Cirillo SL, Subbian S, Chen B, Weisbrod TR, Jacobs WR Jr, Cirillo JD. Protection of Mycobacterium tuberculosis from reactive oxygen species conferred by the mel2 locus impacts persistence and dissemination. Infect Immun. 2009 Jun;77(6):2557-67. doi: 10.1128/IAI.01481-08. Epub 2009 Apr 6. PubMed PMID:19349422; PubMed Central PMCID: PMC2687327
6. Bah A, Lacarrière C, Vergne I. Autophagy-Related Proteins Target Ubiquitin-Free Mycobacterial Compartment to Promote Killing in Macrophages. Front Cell Infect Microbiol. 2016 May 11;6:53. doi: 10.3389/fcimb.2016.00053.eCollection 2016. PubMed PMID: 27242971; PubMed Central PMCID:PMC4863073
7. Katarzyna Z-R, Suresh S. Autophagic degradation of peroxisomes in mammals. *Biochemical Society transactions*. 2016;44(2):431-440. doi:10.1042/BST20150268
8. Ponpuak M, Delgado MA, Elmaoued RA, Deretic V. Monitoring autophagy during Mycobacterium tuberculosis infection. Methods Enzymol. 2009;452:345-61. doi:

10.1016/S0076-6879(08)03621-5. PubMed PMID: 19200892; PubMed Central PMCID: PMC3725297

9. Glick D, Barth S, Macleod KF. Autophagy: cellular and molecular mechanisms. *The Journal of pathology*. 2010;221(1):3-12. doi:10.1002/path.2697
10. Katarzyna Z-R, Suresh S. Autophagic degradation of peroxisomes in mammals. *Biochemical Society transactions*. 2016;44(2):431-440. doi:10.1042/BST20150268
11. Reyrat JM, Kahn D. Mycobacterium smegmatis: an absurd model for tuberculosis? Trends Microbiol. 2001 Oct;9(10):472-4. PubMed PMID: 11597444
12. King GM. Uptake of carbon monoxide and hydrogen at environmentally relevant concentrations by mycobacteria. Appl Environ Microbiol. 2003 Dec;69(12):7266-72. PubMed PMID: 14660375; PubMed Central PMCID: PMC310020
13. Hett EC, Rubin EJ. Bacterial growth and cell division: a mycobacterial perspective. Microbiol Mol Biol Rev. 2008 Mar;72(1):126-56, table of contents.doi: 10.1128/MMBR.00028-07. Review. PubMed PMID: 18322037; PubMed Central PMCID: PMC2268284
14. Brennan PJ. Structure, function, and biogenesis of the cell wall of Mycobacterium tuberculosis. Tuberculosis (Edinb). 2003;83(1-3):91-7. Review. PubMed PMID: 12758196
15. Kilburn JO, Takayama K. Effects of ethambutol on accumulation and secretion of trehalose mycolates and free mycolic acid in Mycobacterium smegmatis. *Antimicrobial Agents and Chemotherapy*. 1981;20(3):401-404.
16. Chatterjee D. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. Curr Opin Chem Biol. 1997 Dec;1(4):579-88. Review. PubMed PMID:9667898.
17. Sánchez D, Rojas M, Hernández I, Radzioch D, García LF, Barrera LF. Role of TLR2- and TLR4-mediated signaling in Mycobacterium tuberculosis-induced macrophage death. Cell Immunol. 2010;260(2):128-36. doi: 10.1016/j.cellimm.2009.10.007. PubMed PMID: 19919859.
18. Robinson JH, Delvig AA. Diversity in MHC class II antigen presentation. *Immunology*. 2002;105(3):252-262. doi:10.1046/j.0019-2805.2001.01358.x.

19. Ahmad S. Pathogenesis, Immunology, and Diagnosis of Latent *Mycobacterium tuberculosis* Infection. *Clinical and Developmental Immunology*. 2011;2011:814943. doi:10.1155/2011/814943.
20. Oliveira-Nascimento L, Massari P, Wetzler LM. The Role of TLR2 in Infection and Immunity. *Frontiers in Immunology*. 2012;3:79. doi:10.3389/fimmu.2012.00079.
21. Harding CV, Boom WH. Regulation of antigen presentation by *Mycobacterium tuberculosis*: a role for Toll-like receptors. *Nature reviews Microbiology*. 2010;8(4):296-307. doi:10.1038/nrmicro2321.
22. Mortaz E, Adcock IM, Tabarsi P, et al. Interaction of Pattern Recognition Receptors with Mycobacterium Tuberculosis. *Journal of Clinical Immunology*. 2015;35:1-10. doi:10.1007/s10875-014-0103-7.
23. Oviedo-Boyso J, Bravo-Patiño A, Baizabal-Aguirre VM. Collaborative Action of Toll-Like and Nod-Like Receptors as Modulators of the Inflammatory Response to Pathogenic Bacteria. *Mediators of Inflammation*. 2014;2014:432785. doi:10.1155/2014/432785.
24. Kerrigan AM, Brown GD. C-type lectins and phagocytosis. *Immunobiology*. 2009;214(7):562-575. doi:10.1016/j.imbio.2008.11.003.
25. Ragas A, Roussel L, Puzo G, Rivière M. The Mycobacterium tuberculosis cell-surface glycoprotein apa as a potential adhesin to colonize target cells via the innate immune system pulmonary C-type lectin surfactant protein A. J Biol Chem. 2007 Feb 23;282(8):5133-42. Epub 2006 Dec 8. PubMed PMID:17158455
26. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procópio DO, Travassos LR, Smith JA, Golenbock DT, Gazzinelli RT. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol. 2001 Jul 1;167(1):416-23. PubMed PMID: 11418678.
27. Nambi S, Long JE, Mishra BB, et al. The oxidative stress network of *Mycobacterium tuberculosis* reveals coordination between radical detoxification systems. *Cell host & microbe*. 2015;17(6):829-837. doi:10.1016/j.chom.2015.05.008.
28. Xie H, Zheng C. OxyR Activation in Porphyromonas gingivalis in Response to a Hemin-Limited Environment. Bäumler AJ, ed. *Infection and Immunity*. 2012;80(10):3471-3480. doi:10.1128/IAI.00680-12.
29. Sherman DR, Sabo PJ, Hickey MJ, et al. Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. Proceedings of the National Academy of Sciences of the United States of America. 1995;92(14):6625-6629.

30. Katarzyna Z-R, Suresh S. Autophagic degradation of peroxisomes in mammals. *Biochemical Society transactions*. 2016;44(2):431-440. doi:10.1042/BST20150268.

31. Lee MY, Sumpter R, Zou Z, et al. Peroxisomal protein PEX13 functions in selective autophagy. *EMBO Reports*. 2017;18(1):48-60. doi:10.15252/embr.201642443.

32. Makni-Maalej K, Chiandotto M, Hurtado-Nedelec M, Bedouhene S, Gougerot-Pocidalo MA, Dang PM, El-Benna J. Zymosan induces NADPH oxidase activation in human neutrophils by inducing the phosphorylation of p47phox and the activation of Rac2: involvement of protein tyrosine kinases, PI3Kinase, PKC, ERK1/2 and p38MAPkinase. Biochem Pharmacol. 2013 Jan 1;85(1):92-100. doi:10.1016/j.bcp.2012.10.010. Epub 2012 Oct 22. PubMed PMID: 23085266.

33. Kumar A, Farhana A, Guidry L, Saini V, Hondalus M, Steyn AJC. Redox homeostasis in mycobacteria: the key to tuberculosis control? *Expert Reviews in Molecular Medicine*. 2011;13:e39. doi:10.1017/S1462399411002079.

34. Kim J, Kundu M, Viollet B, Guan K-L. AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. *Nature cell biology*. 2011;13(2):132-141. doi:10.1038/ncb2152.

35. Houghton JL, Green KD, Pricer RE, Mayhoub AS, Garneau-Tsodikova S. Unexpected *N*-acetylation of capreomycin by mycobacterial Eis enzymes. *Journal of Antimicrobial Chemotherapy*. 2013;68(4):800-805. doi:10.1093/jac/dks497.

36. Branco MR, Marinho HS, Cyrne L, Antunes F. Decrease of H2O2 plasma membrane permeability during adaptation to H2O2 in Saccharomyces cerevisiae. Biol Chem. 2004 Feb 20;279(8):6501-6. Epub 2003 Nov 26. PubMed PMID: 14645222.

37. Kim KH, An DR, Song J, et al. *Mycobacterium tuberculosis* Eis protein initiates suppression of host immune responses by acetylation of DUSP16/MKP-7. *Proceedings of the National Academy of Sciences of the United States of America*. 2012;109(20):7729-7734. doi:10.1073/pnas.1120251109.

38. Settembre C, Di Malta C, Polito VA, et al. TFEB Links Autophagy to Lysosomal Biogenesis. *Science (New York, NY)*. 2011;332(6036):1429-1433. doi:10.1126/science.1204592.

39. Roczniak-Ferguson A, Petit CS, Froehlich F, et al. The Transcription Factor TFEB Links mTORC1 Signaling to Transcriptional Control of Lysosome Homeostasis. *Science signaling*. 2012;5(228):ra42. doi:10.1126/scisignal.2002790.